REMARKS

Background

The instant application is a continuation-in-part of App. Ser. No. 09/863,179 filed on May 23, 2001, now U.S. Pat. No. 6,780,409, and claims priority to Prov. App. Ser. No. 60/206,281 filed on May 23, 2000.

Claim Objections

The Examiner has objected to claims 43-48 as being elected to non-elected subject matter. Specifically, it is suggested that in view of the rejection of the generic claims, non-elected species should be deleted from the claims. As Applicants point out below, the rejections under both 35 U.S.C. §§ 112 and 103 are improper. As such, pending claims 43-48 are allowable.

Rejection under 35 U.S.C. Sec. 112

The Examiner has rejected pending claims 43-48 under 35 U.S.C. §112 as failing to meet the written description requirement. The claims, as currently pending, recite "a post-transcriptional regulatory element". The Examiner contends that, although the application recites the use of post-transcriptional regulatory elements, the claims "encompass a genus of genetic elements that are not described." According to the Examiner, the claims "cover the use of a genus of genetic elements, while providing a description of only a single post-transcriptional regulatory element." (Office Action p.4). Applicants disagree with the Examiner's characterization and application of the written description requirement.

As the Examiner correctly points out, the Guidelines for Written Description state that "[t]he claimed invention as a whole may not be adequately described if the claims require an essential or critical feature which is not adequately described in the specification *and which is not*

conventional in the art or known to one of ordinary skill in the art." (emphasis added). (MPEP 2163(I)(A)).

Applicants submit that the concept of a post-transcriptional regulatory (or control) element was well known in the art, at least as of the filing date of the parent application, and that one of ordinary skill in the art would know how to use appropriate a post-transcriptional regulatory element in the construction of the vector of the invention. (*See, e.g.*, Context Dependence of Different Modules for Posttranscriptional Enhancement of Gene Expression from Retroviral Vectors, *Molecular Therapy*, Vol. 2, No. 5 (2000) (published six months before the filing date of the parent application) (attached) .

Rejection under 35 U.S.C. § 103.

The Examiner has rejected claims 43-48 under 35 U.S.C. 103(a) as being unpatentable over U.S. 2004/01011514 (Liu et al). This rejection is improper.

Liu et al is a published patent application. As of the date of this response no patent has issued based on the Liu application. Liu et al. published in May 27, 2004. The application was filed on May 1, 2003 and is a continuation-in-part of application Ser. No. 09/804,898 that was filed on March 13, 2001. The cited reference claims priority to a provisional application No. 60/189,110 filed in March 14, 2000.

A 35 U.S.C. § 103 rejection must be based on 35 U.S.C. §§ 102(a), 102(b), 102(e), etc. depending on the type of prior art reference used and its publication or issue date. (MPEP 2141.01).

"U.S. patent application publications are prior art under 35 U.S.C. 102(a) and 102(b) as of the publication date. Under amended 35 U.S.C 102(e)(1), a U.S. patent application publication under 35 U.S.C. 122(b) is considered to be prior art as of the *earliest effective U.S. filing date of the published application.*" (MPEP 901.03). It is well established that the effective filing date of a CIP

is the date that the relevant material first appears in the application. (MPEP 2136.02) ("subject matter not included in the parent... can only be used when that subject matter become public."

As the Liu et al. reference published (May 27, 2004) AFTER the filing date of the instant application (March 16, 2004), the reference cannot be prior art under 35 U.S.C. §§ 102(a) or 102(b). As no patent has issued, Applicants assume that the Examiner is citing the reference as prior art under 35 U.S.C. § 102(e)(1), namely, as "an application for patent, published under section 122(b) by another filed in the United States before the invention by the applicant for patent" (35 U.S.C. § 102(e)(1)).

As mentioned above, the Liu et al was filed on May 1, 2003 and is a continuation-in-part of App. Ser. No. 09/804,898 filed in March 13, 2001. The claimed subject matter, namely a vector for expressing GAD in cells of the central nervous system comprising a tissue specific promoter operably linked to a nucleotide sequence encoding GAD and a post-transcriptional regulatory element is disclosed throughout the instant application (See, e.g., paragraph [0023]). The same description also appears in the PARENT application, Ser. No. 09/863,179, that was filed on May 23, 2001 (See, e.g., Column 3 line 66-Col. 4, line 3 of U.S. Pat. No. 6,780,409). Thereby establishing an effective filing date of the material in the instant application of on or before May 23, 2001.

According to the Examiner, the Liu et al reference discloses an AAV vector comprising a Chicken beta-actin (CBA) promoter and a woodchuck post-regulatory element (WPRE) (paragraph 166). The Examiner further states that the the vectors of the (Liu et al) invention can comprise a nucleotide sequence encoding GAD for Expression of GAD in the brain of Parkinson's patients (Para. 149). The Examiner further notes that para. 42 of Liu describes the use of tissue specific promoters with the woodchuck post-regulatory sequence.

The Examiner is correct that Liu et al., as cited, contains a similar recitation as present in the instant application. However, as pointed out above, the Liu reference that published on May 27, 2004 and was filed on May 1, 2003, is a continuation-in-part of a parent application, App. Ser. No. 09/804,898 filed on March 13, 2001 (Liu Parent Application). What the Examiner has failed

to appreciate is that the PARENT application of Liu et al. contains no such similar recitation and that the portions of the reference that the Examiner relies upon for her argument only appear in the latter cited application and therefore have an effective filing date of May 1, 2003 not March 13, 2001.

The Liu Parent Application contains (a) no mention of Glutamic Acid Decarboxylase (GAD); (b) no mention of a post-transcriptional regulatory element (c) no mention of the Chicken beta-actin (CBA) promoter. Consequently, as mentioned above, the effective filing date of the matter that the Examiner relies upon for prior art purposes must be May 1, 2003 -- almost two full years AFTER the effective filing of the instant application. As such the cited reference cannot be deemed to be prior art as against the instant application.

Applicants therefore believe that the claims as they currently stand are allowable. As such allowance is respectfully requested.

Conclusion

Applicants believe that the presently pending claims are in immediate condition for allowance and allowance is therefore respectfully requested. However, should any issues remain, the Examiner is urged to telephone the undersigned Attorney for Applicant in the event that such a communication is deemed to expedite allowance of this application.

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Respectfully submitted,

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ARTICLE

Context Dependence of Different Modules for Posttranscriptional Enhancement of Gene Expression from Retroviral Vectors

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We present a systematic comparison of three modules that enhance expression from retroviral gene transfer vectors at a posttranscriptional level: (i) splice signals (SS) that create an intron in the 5′ untranslated region; (ii) constitutive RNA transport elements (CTE), originally discovered in D-type retroviruses; and (iii) the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE). Here we show that enhancement of expression depends not only on the specific element, but also on the gene of interest, implying context-dependent activity of the RNA elements. Interestingly, different results were obtained for genes that normally require or do not require such control elements. Expression of the HIV-1 *gag-protease* gene, which normally depends on the viral export factor Rev, was strongly enhanced by an oligomeric CTE, while WPRE had only a marginal effect. On the other hand, both CTE and WPRE compensated for the lack of an intron in the expression of human β-globin. In this case, the strongest stimulation of RNA production was observed when functional SS were combined with the WPRE. Both CTE and, in particular, WPRE also enhanced expression of cDNAs that do not normally require any such element (green fluorescent protein, human multidrug resistance-1). In this study, functional SS and WPRE acted in an additive manner, resulting in a 10-fold higher level of expression. Our results indicate that the described modules act on different levels of RNA processing, transport, and translation and that the correct choice of a posttranscriptional enhancer configuration depends on the type of cDNA to be expressed.

Key Words: CTE; WPRE; splice signals; retroviral vector; gene transfer.

Introduction

Effective expression of protein-coding transgenes is a common requirement in experimental molecular biology and gene therapy. To reach this goal, sequence modifications can be introduced that act either at the level of transcription, posttranscriptional processing of nuclear and cytoplasmic RNA, or the half-life and activity of the encoded protein. Given the observation that the majority of transcribed RNA is not efficiently processed and licensed for nuclear export (1), and that cytoplasmic accumulation and translation of many cellular RNAs is rate-limiting and can be dependent on the presence of appropriate introns, export signals and/or polyadenylation tails (2, 3), there is a growing interest in sequences that improve posttranscriptional processing and transport of a given RNA.

At least three different cis-acting modules have been shown to be of exceptional importance in this context: splice sites (SS), retroviral constitutive RNA transport elements (CTE) and hepadnaviral posttranscriptional regulatory elements. SS are of particular value when located in the 5′ untranslated region (5′UTR) of transgenic RNA (3, 4). Splicing may promote mRNA export (3, 5–7) and translation (4), and protein expression is commonly

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dependent on successful completion of the splicing process.

Retroviral replication, on the other hand, requires utilization of incompletely and unspliced RNA and many retroviruses have evolved mechanisms to circumvent the requirement for splicing in nuclear export of RNA. In the case of the complex retrovirus human immunodeficiency virus type 1 (HIV-1), nuclear export of incompletely spliced mRNAs is mediated by the Rev/RRE system, where the viral encoded Rev-protein binds *in trans* to a cis-acting sequence termed the Rev-responsive element (RRE) present on intron containing mRNAs (8). In simple retroviruses such as Mason–Pfizer monkey virus (MPMV), the efficient cytoplasmic accumulation of unspliced mRNA is dependent on a CTE (9), which is a cis-acting RNA element that interacts directly with cellular factors such as the tip-associated protein, TAP (10, 11). CTEs have also been observed in other D-type retroviruses and functionally related modules have been detected in the genomes of some mouse intracisternal A-type particles (IAP) and foamy viruses (12, Wodrich *et al.*, submitted for publication). The presence of a CTE, or even better, oligomeric copies of the CTE, allows expression of an unspliced RNA that contains sequences which otherwise inhibit nuclear export (9, 13). In contrast to the functionally distinct Rev/RRE system of HIV-1, the CTE is active in absence of virus-encoded proteins (reviewed in 8, 14). Interestingly, functionally related elements have been identified in intronless cellular or viral protein-coding transcripts, e.g., histone H2a (15) or herpes simplex virus thymidine kinase (16).

Besides SS and CTE, the third RNA element that can significantly enhance transgene expression is the post-transcriptional regulatory element of hepadnaviruses, such as hepatitis B virus (17) or woodchuck hepatitis virus (WPRE) (18), the latter being more efficient (19, 20). The WPRE seems to act independent of transcription and splicing and may improve gene expression by modification of polyadenylation, RNA export, or translation (2, 18 and unpublished data cited in 20).

Several studies, including our own, have demonstrated the utility of these three groups of elements for increasing transgene expression in context of either plasmid or viral gene vectors (3, 4, 12, 18–23, Wodrich *et al.*, submitted for publication, and references therein). However, no systematic and comprehensive evaluation of these RNA elements has been performed to date and a potential interdependence of their activities as well as their potential for synergism has not been analyzed. Furthermore, the relative role of the gene of interest has also not been evaluated. Such a systematic analysis is the prerequisite for the rational design of improved expression vectors.

In the present study, we addressed these issues in the context of retroviral vectors that mediate stable insertion of transgenes in chromosomal DNA, using four different cDNAs that are of interest both in experimental molecular biology and in gene therapy: a fragment of human immunodeficiency virus type 1 (HIV-1) comprising the *gag* and *protease* genes (*gpII*), human *β-globin,* enhanced green fluorescent protein (*EGFP*), and human multidrug resistance-1 (*MDR1*). These cDNAs were chosen because, with respect to RNA processing, they belong to three classes of functionally distinct indicator genes. Cytoplasmic accumulation of the HIV *gpII* transcript, which contains strong nuclear retention signals (24, 25), is known to be dependent on presence of Rev/RRE or a CTE (reviewed by 8, 14). Expression of *β-globin* requires splicing of an intron for RNA export (26), whereas expression of *EGFP* and *MDR1* is possible, although suboptimal, in the absence of a CTE or an intron (23). Our analysis clearly shows that the function and potential synergy of the RNA elements tested is dependent on the type of cDNA to be expressed, indicating a potential regulatory hierarchy in cellular decisions on RNA processing. Inclusion of appropriate RNA elements may determine whether a given transcript can be expressed at all, or mediate a relative enhancement of expression in the range of more than one order of magnitude. A better understanding of the underlying mechanisms combined with a comprehensive side-by-side evaluation provides a rational approach for improving expression of a given transgene by incorporation of RNA elements.

Materials and Methods

Plasmids. Vectors MP110, MP11, MP71, and MP91 are derivatives of SF110, SF11, SF71, and SF91, respectively (23). These vectors contain LTRs from myeloproliferative sarcoma virus (MP) or friend spleen focus-forming virus (SF) and have modifications within the untranslated leader region, which in all cases is devoid of *gag*-sequences (Fig. 1). In leader 110 the retroviral splice donor present in leader 11 has been deleted by site-directed mutagenesis (23). Leader 71 is based on leader 11 and contains in addition a minimal viral splice acceptor to create a full intron (0.4 kb in size). Leader 91 is similar to leader 71, but carries a mutation in a ATG site located between the SS. All vectors contain a *NotI*, *Eco*RI, *Bam*HI, *Hin*dIII multiple cloning site (MCS) in which we inserted the transgenes and RNA elements (Fig. 1).

GpII (HIV-1 gag/prot) was used as an *Eco*RI fragment derived from pK-R-gpII (27). GpII contains part of the 5'UTR, the complete *gag* gene and the protease (PR)-coding region of the *pol* gene of HIV-1, strain BH-10 (28).

The EGFP cDNA was obtained from pKS-EGFP-I as a *NotI*/*Eco*RI fragment. In pKS-EGFP-I the EGFP cDNA (Clontech) is fused to an epitope-tag and a his-tag (kindly provided by R. Welker).

SF91m3 contains the 3.9-kb *MDR1* cDNA as a *NotI*/*Bam*HI fragment, with a silent mutation in the cryptic splice acceptor at bp 2.319 (29).

The *β-globin* cDNA was created by reverse transcriptase PCR of RNA isolated from full blood of a healthy donor. The following primers were used for amplification: globin5, 5'-GGGCGGCCGCGGTACCATGGTG-CACCTGACTCCTGAGG-3', which harbors a *NotI* site (underlined); and globin3, 5'CCGAATTCAGGCGTAGTCGGGCACGTCGTAGGGGTAGT-GATACTTGTGGGCCAGGGC-3', creating a HA epitope tag at the 3' end, followed by an *Eco*RI site (underlined). Sequencing revealed two silent point mutations according to the GenBank human *β-globin* mRNA sequence (Accession No. V00497).

WPRE and CTE sequences have been mapped by the Mulfold program (30, 31) and were amplified accordingly by PCR, sequenced and inserted into the MCS of the retroviral vectors immediately 3' of the cDNAs. The IAP element was amplified from pMIA14 (GenBank Accession No. M17551) using primers 5'MIAminforXho (5'-TCTC-GAGTCAGAGCCAGATAGGCCCAATG-3') and 3'MIAminrevClaNhe (5'-

TGGGCTAGCATCGATAGAGCCAAGGCTGTCTG-3'). The MPMV CTE (nt 8006–8175) was derived from clone SIVMPCG (GenBank Accession No. M12349), kindly provided by E. Hunter (Birmingham, AL), using primers 5'CTE (5'-GCTCTAGAGCCAGATAGGCCC-3') and 3'CTE (5'-AAAGCTTGCTAGCTGATCAACACATCCCTCGGAGGC-3'). The Foamy element was cloned by amplification of nucleotides 6128 to 6683 from the human foamyvirus sequence (HSRV GenBank Accession No. U21247) with primers 5'Fo (5'-GGTCTAGAAACAACACCTATAGCCC-3') and 3'Fo (5'-GGGCTAGCACAACAAGTATAAAGC-3'). The 4xCTE was obtained from 3-CCCC (13). A 600-bp WPRE fragment was amplified from pWHV8 (NCWHV, EMBL Accession No. J02482, nt 901–1501, kindly provided by H. Will, Hamburg) using primers 5'WPRE (5'-GGAGGCC-TATCGATGAATTGCAGCATCTTACCGCCATTTATTCCC-3') and 3'WPRE (5'-GGAGGCCTATCGATGAATTCGAAAGGACGTCAGCTTCCCCG-3'). This fragment was subcloned into pKS– (Stratagene) and later inserted into the retroviral vector as a *Bam*HI/*Hin*dIII fragment.

Cells and transfections. Amphotropic Phoenix (32) packaging cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, and 2 mM glutamine. The day before transfection, 2×10^6 cells were plated on a 6-cm dish. For transfection, the medium was exchanged and 25 μM chloroquine (Sigma) was added. 8 μg retroviral vector DNA, and 1 μg of a EGFP reporter plasmid (unless EGFP was part of the retroviral vector) to determine transfection efficiencies, was transfected using the calcium phosphate precipitation method. To generate VSV-G pseudotyped particles, 4 μg M13 (MuLV gag/pol expression plasmid) and 2 μg M3 (VSV-G expression plasmid) were transfected in addition. Medium was changed after 10–12 h. Equal transfection efficiency was either controlled by counting EGFP positive cells or by FACS analysis of transfected cells. Supernatants containing the viral particles were collected 36–60 h after transfection, filtered through a 0.45-μm filter, and used to transduce 5×10^4 target cells. Transduction was assisted by adding 8 μg/ml polybrene and centrifugation for 90 min at 2000 rpm and 25–32°C. In case of retroviral vectors expressing EGFP, vector titers were determined by transducing predefined numbers of target cells with serial dilutions of supernatant and analyzing the percentage of positive cells by flow cytometry. Infectious titers were in the range of $1–5 \times 10^6$ transducing units/ml on NIH3T3 cells. In all other cases, transduced cells were analyzed by indirect immunofluorescence for expression of the respective gene of interest. Further analysis was limited to those experiments where less than 40% of target cells were productively transduced, because higher transduction rates may lead to multiple integration events. Transduction experiments were performed with mouse NIH3T3 (fibroblasts), human SW480 cells (colon carcinoma), canine Cf2th (thymus epithelium), and human K562 (chronic myeloid leukemia). Except for K562 (cultured in RPMI 1640), all cell lines were cultured in the medium described above. After transduction, cells were grown for 5–6 days and subsequently analyzed by flow cytometry, fluorescence microscopy, Western blot, and Northern blot.

Immunofluorescence staining and microscopy. Cells grown on cover slips were fixed with methanol/acetone (1:1) at –20°C for 10 min and air dried. MDR1 P-glycoprotein was detected using 10 μl UIC2-PE antibody (Immunotech), diluted 1:100 in PBS containing 10% FCS. Immunostaining was performed for 1 h in a 37°C moist chamber. Fluorescent cells were scored on a Zeiss Axioscop fluorescence microscope.

Western blots. Cell lysates normalized for protein levels were fractionated on SDS–polyacrylamide gels containing 17.5% polyacrylamide (200:1 ratio of acrylamide to *N,N*-methylenbisacrylamide). Following electrophoresis, the proteins were transferred for 45 min at 4°C to nitrocellulose membrane (0.45 μm, Schleicher and Schuell). The membrane was blocked with 10% dry milk in PBS for 1 h and stained with antiserum against HIV-1 CA (diluted 1:2000 in 5% dry milk in PBS containing 0.5% Triton X-100) overnight, followed by an additional 1 h blocking step and incubation with peroxidase conjugated anti rabbit secondary antibody (Dianova; diluted 1:10,000 in 5% dry milk in PBS containing 0.5% Triton

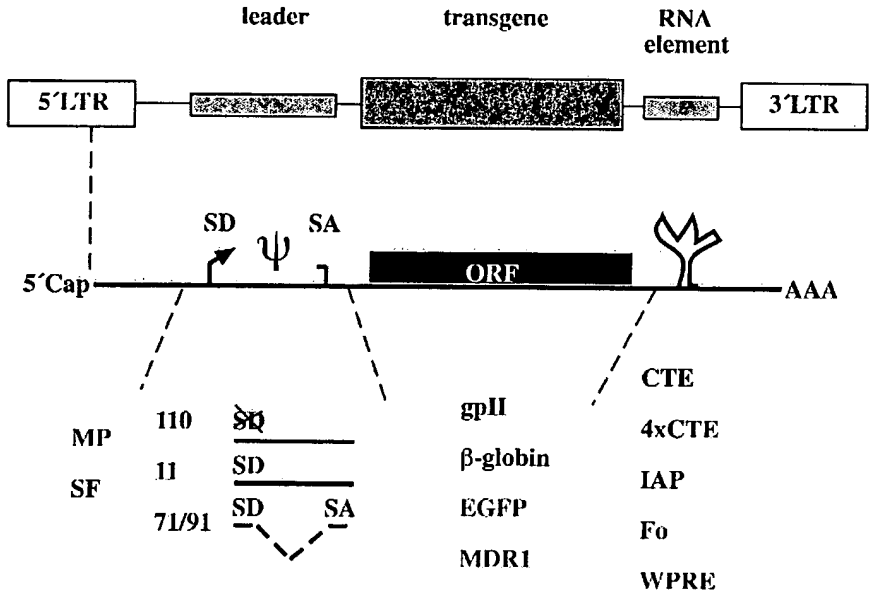

FIG. 1. Schematic design of retroviral vectors used in this study. The scheme shows the modular composition of retroviral vectors with the respective LTRs, splice context in the 5' untranslated leader region, transgene, and posttranscriptional control elements inserted into the 3' untranslated region. LTRs were derived from myeloproliferative sarcoma virus (MP) or Friend spleen focus-forming virus (SF). The *gag*-deleted leader region was derived from the murine embryonic stem cell virus, and contained either no splice sites (leader 110), a splice donor (SD) (leader 11), or a SD in combination with a splice acceptor (SA) (leader 71 or leader 91), creating an intron encompassing the packaging signal (Ψ); cDNAs used were either HIV-1 *gag* (*gpII*), human *β-globin, enhanced green fluorescent protein* (*EGFP*), or human *multidrug resistance 1* (*MDR1*). Posttranscriptional control elements were either the constitutive transport element (CTE) from the Mason–Pfizer monkey virus or four copies thereof (4xCTE), elements from an intracisternal A-type particle (IAP) and from the human foamyvirus (Fo), or the posttranscriptional regulatory element from the Woodchuck hepatitis virus (WPRE).

X-100) for 2 h. Detection was performed by enhanced chemiluminescence (Amersham) according to the manufacturer's protocol. Blots were exposed to Kodak X-Omat-AR films.

Flow cytometry. At least 10^6 cells were harvested and washed in PBS. To determine expression of the MDR1 transgene, cells were incubated for 30 min with monoclonal antibody UIC2– (Coulter–Immunotech), washed twice with PBS, treated with a goat anti-mouse FITC-conjugated secondary antibody (Immunotech), again washed with PBS, and analyzed in a FACScalibur (Becton–Dickinson) using CellQuest software (Becton–Dickinson). A gate was set on a homogenous cell population, as determined by scatter characteristics, and at least 25,000 events were monitored. A marker was set to calculate the percentage and mean fluorescence intensity of positive cells.

Northern blot. Total RNA preparation was performed using the RNAzol extraction method (Wak Chemicals) as previously described (13). Five micrograms of each RNA was separated at 0.6 V/cm^2 for 4 h in denaturing formaldehyde gels. Subsequently RNAs were transferred to Biodyne B membrane (0.45 μm, Pall) by capillary transfer, UV crosslinked (Stratalinker), and heat fixed for 15 min at 80°C. Transfer efficiency was determined by staining the blot for 5′ with methylene blue (0.04% in 0.5 M NaOAc, pH 5.2). Hybridization was performed using standard procedures as previously reported (13). Specific probes (25 ng) corresponding to the 3′UTR of the respective retroviral vector were radiolabeled using the Prime It II kit (Stratagene) to an activity of at least 10^6 cpm/ml hybridization solution and separated from unincorporated nucleotides on spin columns (Mobitec). Filters were washed, sealed, and exposed to X-ray films (Kodak X-Omat-AR) or quantified by Phosphoimager (Fuji) analysis.

RESULTS

Construction and Generation of Retroviral Vectors

To investigate interactions of the three classes of post-transcriptional regulators (SS, CTE and related sequences, WPRE) with distinct types of cDNAs, we constructed retroviral vectors lacking viral coding sequences that could possibly interfere with the function of the inserted cDNAs (23). An additional advantage of this vector series for the purpose of the present investigation is their modular assembly allowing precise exchange of cis-active elements, the existence of variants with defined alterations in SS located in the 5′UTR (Fig. 1), and the high-titers obtained upon transient transfection into Phoenix packaging cells (32; Wahlers *et al.*, submitted for publication). The CTE and WPRE elements were inserted immediately 3′ of the cDNAs, and thus in the 3′UTR upstream of the retroviral 3′LTR, which contains the polyadenylation signal.

These plasmids were transfected into Phoenix packaging cells for transient production of retroviral vector stocks. Transfection efficiency was controlled by cotransfection of EGFP plasmids when using retroviral vectors expressing gpII or β-globin, or directly by flow cytometry of retroviral transgene expression in Phoenix cells (data not shown). For identical plasmids, titers of cell-free retroviral vector stocks harvested after independent transfections of Phoenix packaging cells were highly reproducible. In the case of EGFP-expressing vectors, titers measured by flow cytometry of transduced NIH3T3 cells were in the range of 1–5 × 10^6 infectious units per milliliter, depending on the RNA element. Generally, the lowest titers were obtained with splice-defective vectors (leader 110), the highest with vectors harboring the retroviral splice-donor but lacking the retroviral splice acceptor (leader 11), and intermediate levels with vectors containing a full, alternatively spliced intron surrounding the retroviral packaging signal (leader 71 or 91). Similar findings had been obtained previously with other cDNAs and packaging cells (23). Presence of the other RNA elements (CTE, 4xCTE, IAP, Foamy element, or WPRE) altered vector titers only slightly in context of MDR1 or EGFP (up to twofold), but had a significant influence on vector titers in context of gpII or β-globin (see below).

Enhancing Expression of a cDNA Harboring Nuclear Retention Signals: CTE Is Clearly Superior to WPRE

Expression of the HIV-1 structural proteins is known to be dependent on presence of the Rev/RRE system for RNA export, which can be functionally replaced by CTE-sequences of D-type retroviruses (9, 33). Thus, *gpII* comprising HIV-1 *gag* and part of the *pol* gene is an ideal reporter to analyze the effect of different posttranscriptional control elements on a Rev-dependent transcript. Using transient transfection assays, we recently showed that other retroviral elements located in the *pol/env* region of an IAP and of human foamy virus could substitute for Rev/RRE in eukaryotic expression vectors, similar to the CTE of MPMI (Woldrich *et al.*, submitted for publication; 13). Here, we extended these observations using retroviral vectors, thereby assessing the function of these different types of CTE and related sequences in the context of chromosomally integrated genes.

Transductions of target cells with retroviral vectors expressing *gpII* were performed without adjusting viral titers. Therefore, expression differences shown with *gpII* reflect a combination of two variables: (i) differences in gene dosage, because altered processing and nuclear export of vector RNA in the transfected packaging cells can influence vector titer; and (ii) differences in post-transcriptional RNA processing and export in the retrovirally transduced target cells. The effects on vector titer and on transgene expression are both determined by the relative efficiency of the respective element, and scoring cumulative effects therefore appears to be legitimate to reveal differences between various RNA modules.

Western blot analysis of retrovirally transduced canine Cf2th cells (Fig. 2A) showed that expression of HIV-Gag protein required the presence of a CTE (Fig. 2A, lane 3) or of a corresponding activity like the RNA element derived from IAP (not shown) or Foamy virus (Fig. 2A, lane 5). Although the sequences derived from IAP and Foamy virus acted as efficiently as RNA expression elements in transient transfection assays (Woldrich *et al.*, submitted for publication; 13), they were less efficient than the CTE following retroviral transduction. Highest expression of HIV-Gag was achieved with the oligomeric CTE (Fig. 2A, lane 4). The WPRE, on the other hand, had no significant effect (Fig. 2A, lane 6), indicating that this

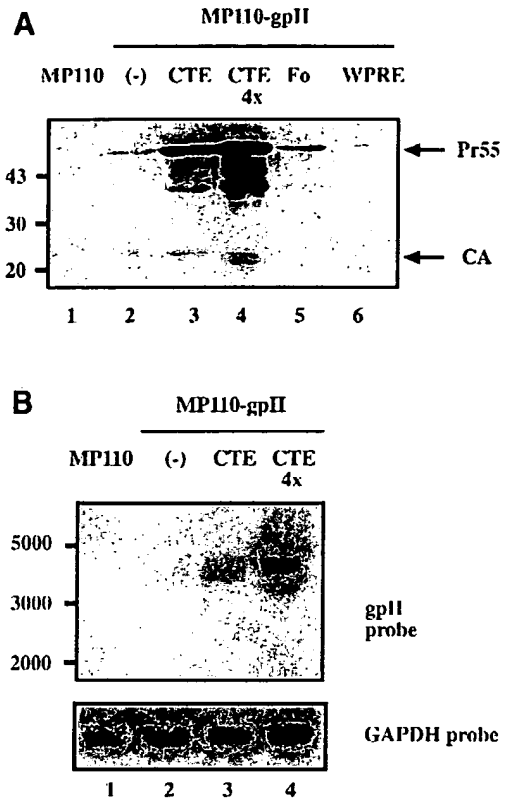

FIG. 2. Posttranscriptional control elements modulate HIV-1 Gag expression. (A) Western blot analysis of transduced Cf2th cells: Lanes 1–6 show cell lysates of Cf2th cells transduced with retroviral vectors MP110 (lane 1), MP110-gpII without RNA element (lane 2) or derivatives of MP110-gpII containing posttranscriptional control elements as indicated above each lane (lanes 3–6). Arrows point to the Gag polyprotein (Pr55) and its cleavage product, the capsid (CA) protein. Additional bands correspond to HIV-1 Gag cleavage intermediates. Molecular mass standards in kDa are given on the left. (B) Northern blot analysis of transduced SW480 cells: Transduction was performed with retroviral vectors MP110, MP110-gpII (–), or derivatives of MP110-gpII containing the CTE or four copies thereof (4xCTE). Gag specific RNA was detected with a radiolabeled probe corresponding to the complete *gag/PR* coding region present in all MP110-gpII derived vectors. Molecular size standards in bp are given on the left. The blot was rehybridized with a radiolabeled probe corresponding to GAPDH.

element is functionally not equivalent to a CTE. Similar results were obtained in SW480 cells.

Northern blot analysis of total RNA preparations from transduced cells (Fig. 2B) revealed significantly increased mRNA levels for the CTE bearing transgene (Fig. 2B, lane 3), which was even higher in the presence of multiple copies of the CTE (Fig. 2B, lane 4). Moreover, the higher molecular weight of the transcript carrying four copies of the CTE suggests that oligomerization of this sequence was preserved after reverse transcription.

Enhancing Expression of a cDNA Requiring Active Splicing for Export: CTE and WPRE Compensate for the Absence of an Intron

Several investigators have shown that the *β-globin* message requires splicing of an intron in the 5'UTR for efficient cytoplasmic expression (2, 21, 26). We were interested to determine whether a retroviral intron, generated by splice signals flanking a minimal retroviral packaging sequence (23), could be used instead of the genomic intron for expression of *β-globin* from a retroviral vector, and to what extent other RNA elements would further enhance expression of *β-globin*. To this end, we inserted the *β-globin* cDNA into the splice-defective vector, MP110, and into its counterpart harboring functional retroviral SS in the 5'UTR, MP71. As with *gpII*, we also inserted the different RNA elements into the 3'UTR (Fig. 1). The performance of the different vectors was tested in the absence of selection and without adjusting the m.o.i. Northern Blot analysis of transfected packaging cells indicated significant differences in RNA-levels depending on the RNA element (data not shown), thus confirming that we measured a combined effect of the RNA elements on vector titer and on expression in target cells.

Northern blot analysis of transduced SW480 cells revealed that the WPRE and, surprisingly, also the CTE compensated for the absence of an intron (Fig. 3,lanes 2 and 3), whereas the elements derived from IAP or Foamy virus showed no significant effect (data not shown). Of note, retroviral SS were equally efficient to restore expression of *β-globin* (Fig. 3, compare lanes 2 to 4). However, a remarkable increase in expression was observed when SS were combined with the WPRE, indicating synergy or at least additive cooperation of these elements for expression of *β-globin* (Fig. 3, lane 6). Although to a lesser extent, cooperation was also observed for the combination of SS with the CTE (Fig. 3, lane 5). Interestingly, cells transduced with the CTE containing vector expressed a substantial amount of unspliced RNA (Fig. 3, lane 5), whereas in the presence of the WPRE the predominant signal corresponded to the spliced RNA (Fig. 3, lane 6), as verified by comparison of size.

Enhancing Expression of a cDNA Requiring Neither CTE nor Intron for Export: WPRE and SS Are Superior to CTE

The data accumulated so far indicated that the functions of WPRE and CTE are only partially overlapping and are highly dependent on the context of neighboring RNA control elements of the transcription unit. While *gpII* and *β-globin* may act as paradigms for CTE-dependent and splice-dependent transcripts, respectively, many other cDNAs can be expressed in absence of SS or a CTE. For such sequences, RNA elements may still mediate a relative enhancement of expression. Therefore we investigated the impact of SS, CTE, or WPRE, individually and in combination, on expression of *EGFP*, which can be precisely monitored in unselected cells by flow cytometry. In this case, experiments were performed at equivalent m.o.i. values, and thus with equivalent gene dosage, because EGFP vector titers could be directly determined by flow cytometry of transduced cells (Wahlers *et al.*, submitted for publication; 34).

Vectors MP110-EGFP (deleted splice donor), MP11-

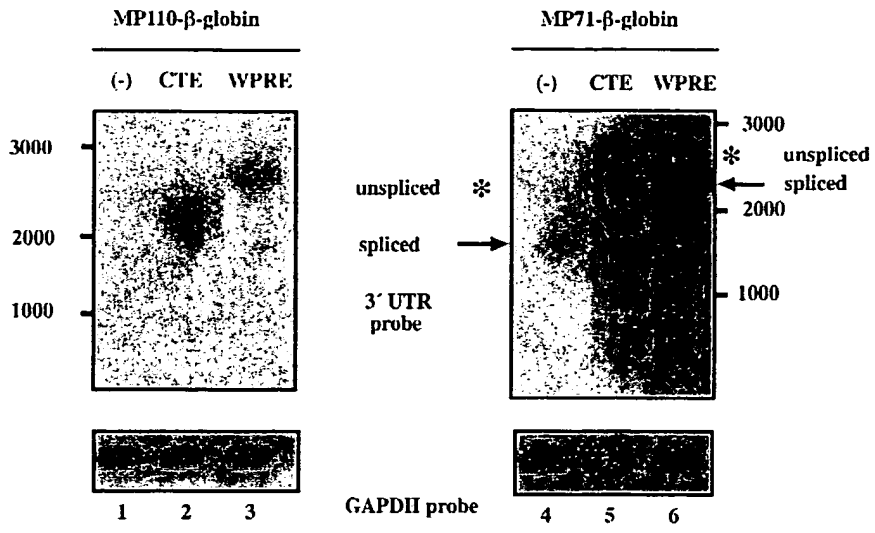

FIG. 3. Splice signals plus WPRE or CTE mediate strong enhancement of *β-globin* expression. Northern blot of RNA preparations from unselected, polyclonal populations of SW480 cells transduced with the vectors indicated above each lane. The radioactive probe corresponded to the 3'MPSV LTR present on all transcripts. Unspliced and spliced RNAs derived from MP71-β-globin are indicated with asterisks and arrows, respectively. GAPDH levels are shown below.

EGFP (active splice donor), and MP71-EGFP (full intron) were used to transduce target cells. Northern blot analysis of total RNA preparations from polyclonal, unselected populations of transduced SW480 cells showed the unspliced transcript when no SS were present (Fig. 4A, lane 2). Compared to the splice donor-deleted transgene (MP110-EGFP), the presence of an active splice donor (MP11-EGFP) led to a slight increase (1.2×) in steady-state RNA levels (Fig. 4A, lanes 2 and 3) and to a similar stimulation of protein expression, as determined by mean fluorescence in FACS analysis of transduced cells (Fig. 4B, columns 1 and 7). The presence of a functional intron (vector MP71-EGFP) resulted in efficient splicing (Fig. 4A, lane 4), concomitant with a twofold increase of RNA levels and an almost threefold increased mean fluorescence (Fig. 4B, column 10; Table 1). This indicates a dual effect of 5'UTR splicing upon transgene expression, firstly at the level of RNA export and/or stabilization and secondly at the level of translation.

Next, we tested the influence of different posttranscriptional control elements (IAP element, CTE, 4xCTE, Foamy element, WPRE) in context of the splice-defective vector, MP110-EGFP, as well as in context of the splice-competent vector, MP71-EGFP. In contrast to our observations with *gpII* (Fig. 2) and *β-globin* (Fig. 3), there was no increase in *EGFP* expression in the presence of either the IAP element or the CTE (or multiple copies thereof), independent of the splice context (Fig. 4B, columns 2 to 4 and data not shown). However, the Foamy element, and more significantly, the WPRE were capable of enhancing *EGFP* expression (Fig. 4B, columns 5, 8, 11 and 6, 9, 12). Northern blot analysis revealed that the Foamy element and the WPRE acted on both, spliced and unspliced message, with no significant effect on splicing (Fig. 4A, lanes 8 to 10). However, the Foamy element was significantly weaker than the WPRE. In addition, the WPRE acted as an RNA enhancer both in splice-defective and splice-competent vectors, whereas the Foamy element (and other CTE related elements, data not shown) had no effect in splice-competent vectors (Fig. 4A, lane 9; Fig. 4B, column 12, and Table 1). As with *β-globin*, the highest increase in expression (more than tenfold compared to the basic construct MP110-EGFP) was observed with the combination of WPRE and SS (Fig. 4A, lane 10; Fig. 4B, column 12, and Table 1). Interestingly, the effect of the WPRE was weaker in the context of the splice-competent vector (MP71) than in the context of the splice-deficient vector (MP110), resulting in a 50% reduction of WPRE-enhancement in the splice-context, both at RNA and protein levels (Table 1). These results indicate a partial overlap in the function of SS and WPRE.

Combinations of other elements (e.g., Foamy element and CTE, Foamy element and WPRE) always resulted in intermediate expression levels, roughly equivalent to the mean of the values recorded for each element alone (data not shown). This argued against cooperation for these combinations of elements.

A side-by-side comparison of Northern blot data and flow cytometry revealed that analysis of cellular fluorescence was an accurate reporter of RNA expression. While in most cases enhancement of RNA expression and protein levels, as detected by cellular fluorescence, were equivalent, we reproducibly noted that the WPRE (and also the intron, see above) produced an even stronger increase in protein than in RNA levels, indicating a function not only in nuclear RNA processing and export, but also in translation (Table 1).

To exclude that relative enhancement of expression levels was a consequence of differences in transgene copy numbers, Cf2th cells were transduced with serial dilutions of supernatants from MP110-EGFP, MP110-EGFP-Fo, and MP110-EGFP-WPRE, and mean fluores-

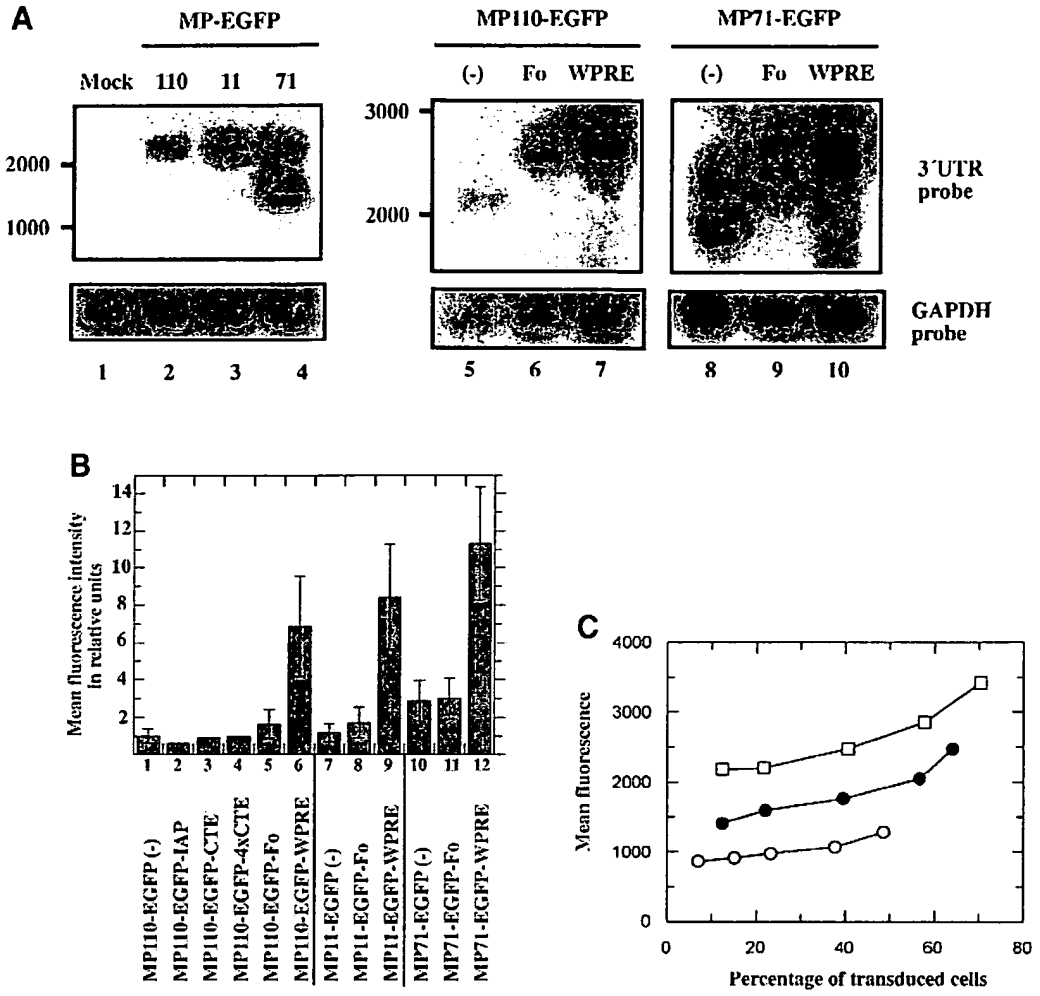

FIG. 4. Expression levels of EGFP from a retroviral vector can be modified by posttranscriptional control elements. (A) Posttranscriptional control elements lead to enhanced RNA levels. Northern blot analysis of EGFP transcripts in SW480 cells transduced at equivalent m.o.i. with the retroviral vectors indicated. Asterisks correspond to additional bands resulting from splice products. The relative increase of retroviral RNA expression dependent on presence of splice sites or other RNA elements was quantified by Phosphoimager analysis (see Table 1). All blots were hybridized as described in Fig. 3. (B) Combinatorial effects of splice context and RNA elements in enhancing EGFP expression: The mean fluorescence intensity and standard deviations determined by flow cytometry of unselected SW480 cells are shown for three independent transductions with the vectors indicated. The activity obtained for the vector lacking splice sites and RNA elements was arbitrarily set as 1. (C) Influence of the number of integrated vector genomes on EGFP expression. Cf2th target cells were transduced with five serial dilutions (1:1, 1:2, 1:4, 1:8, and 1:16) of vector containing supernatants from Phoenix cells transfected with MP110-EGFP (○), MP110-EGFP-Fo (●) and MP110-EGFP-WPRE (□). Mean fluorescence intensity of transduced cells (y-axis) and percentage of transduced cells (*x*-axis) was analyzed by flow cytometry 5 days posttransduction. Productive transduction of more than 30% of target cells is associated with an increased probability for the presence of cells harboring >1 transgene integration (34, 36). Similar results were achieved with SW480 cells, but the effect of the WPRE was weaker in Cf2Th compared to SW480 cells.

cence values were plotted against percentage of *EGFP*-expressing cells (Fig. 4C). The resulting curve shows that RNA element-dependent enhancement of *EGFP* expression was not influenced by the infectious titer. Since higher transduction levels (>40%) raise the probability of multiple integration events (Wahlers *et al.*, submitted for publication; 36), a slight increase in mean fluorescence is observed with all types of vectors using higher m.o.i. values (Fig. 4C).

Taken together, the results obtained with *EGFP* vectors suggested that highest expression of such transgenes could be achieved with SS in the 5'UTR in combination with the WPRE, whereas a CTE did not act as a strong enhancer. The CTE-related Foamy element was slightly superior to a CTE in absence of SS, but had no effect in presence of SS.

The Combination of SS and WPRE Enhances Expression of the Human Multidrug Resistance Gene

The multidrug resistance gene (*MDR1*) encodes a membrane efflux pump that extrudes a variety of amphiphilic agents from the cytoplasm, including fluorochromes and, clinically more important, cytotoxic anti-cancer drugs.

TABLE 1
Relative Stimulation of RNA and Protein Expression in SW480 Cells Transduced with EGFP Vectors

Vector backbone	Splice context	RNA element	Fold stimulation RNA	Fold stimulation protein
MP110-EGFP	na	na	1	1
MP11-EGFP	SD	na	1.24	1.67
MP71-EGFP	SD/SA	na	2.00	2.815
MP110-EGFP	na	na	1	1
MP110-EGFP	na	Fo	1.93	1.745
MP110-EGFP	na	WPRE	3.93	6.833
MP71-EGFP	SD/SA	na	1	1
MP71-EGFP	SD/SA	Fo	0.8	1.041
MP71-EGFP	SD/SA	WPRE	1.73	3.409

Note. RNA stimulation factors were calculated from phosphoimage analysis of Northern blots normalized for GAPDH levels. Stimulation factors were also normalized for transduction rates, but these varied in the range of only a few percent as determined by flow cytometry. Protein expression levels and the respective stimulation factors were calculated from the mean fluorescence intensity of EGFP-positive cells. Vectors MP110-EGFP and MP71-EGFP containing neither splice sites nor RNA elements were arbitrarily set to 1. na, not applicable.

Thus, *MDR1* is a paradigm for an *in vivo* selectable marker gene which, when highly expressed, may induce resistance to cytotoxic drugs in transgenic cells (35, 36). We previously showed that inclusion of SS in the 5'UTR of a retroviral vector elevates *MDR1* expression significantly, both in hematopoietic cell lines (23) and in primary human hematopoietic cells (29). Here we tested whether expression from such a splice-competent retroviral *MDR1*, SF91m3 (29), could be further improved by including in the 3'UTR a WPRE (vector SF91m3-WPRE) or the Foamy element (vector SF91m3-Fo).

SW480 cells were transduced with these vectors at comparable m.o.i., and polyclonal, unselected populations were analyzed 6 days posttransduction using a monoclonal antibody directed against the *MDR1*-encoded P-glycoprotein. Fluorescence microscopy (Fig. 5A) revealed that transgene expression was clearly increased in cells transduced with the WPRE-vector compared to cells transduced with the standard vector or the vector harboring the Foamy element. The experiment was repeated, and the differences in expression levels were quantified by flow cytometry, revealing a threefold increase in mean fluorescence intensity with the WPRE, whereas neither CTE nor the Foamy element did increase transgene expression (Fig. 5B, Table 2). Functionality of the *MDR1*-encoded efflux pump in transduced cells was verified using the rhodamine 123 efflux assay (data not shown) as described previously (37). Northern blot analysis confirmed elevated expression of *MDR1* RNA dependent on presence of the WPRE (data not shown).

Discussion

To our knowledge, this is the first comparative study presenting a systematic analysis of the impact of three different types of posttranscriptional RNA signals (SS, CTE,

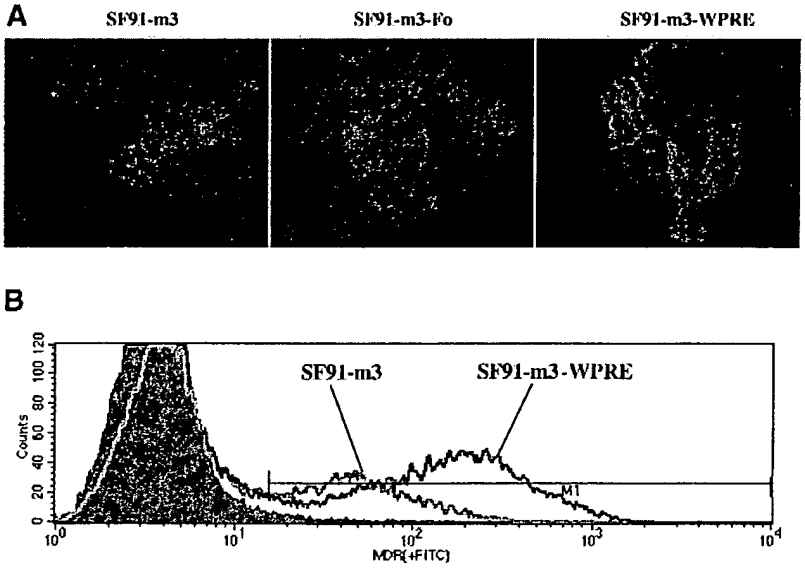

FIG. 5. Posttranscriptional control elements enhance expression of the *MDR1*-gene. (A) Immunofluorescence analysis of SW480 cells transduced with retroviral vectors SF91-m3, SF91-m3-Fo, or SF91-m3-WPRE: Cells were fixed 6 days posttransduction with paraformaldehyde and detection of the *MDR1*-encoded P-glycoprotein was performed using a phycoerythrin (PE)-conjugated monoclonal antibody (UIC2-PE). Transduction rates were similar for all vectors as determined by flow cytometry. (B) FACS analysis of SW480 cells transduced with retroviral vectors SF91-m3 or SF91-m3-WPRE. Marker M1 was set on MDR1-positive cells and mean fluorescence intensities were calculated from this population of cells and are given in Table 2. Cells were stained with monoclonal antibody UIC2 followed by a FITC-conjugated anti-mouse antibody. Cells transduced with the vector MP110 lacking *MDR1* (green) were used as a control.

TABLE 2
Enhancement of MDR1 Expression by RNA Regulatory Elements

Vector	% transduced cells	Mean
SF91-m3	26.50	71.77
SF91-m3-CTE	50.48	72.80
SF91-m3-Fo	44.63	84.65
SF91-m3-WPRE	54.15	230.81

Note. Mean, mean fluorescence intensity of a polyclonal culture of SW480 cells transduced with the respective retroviral vector.

and WPRE) on enhancement of expression of chromosomally integrated transgenes. The performance of these elements was tested either alone or in combination, and in the context of three different types of coding sequences: the *gag* gene of HIV-1 as a paradigm for an export factor-dependent transcript, human *β-globin,* a typical intron-dependent transcript, and cDNAs such as *EGFP* and human *MDR1,* which can be expressed in the absence of a CTE or an intron. All experimental procedures were performed in absence of selection, allowing a qualitative comparison of the utility of the RNA elements under investigation in the context of HIV-1 *gag* and β-*globin,* and more precise quantitative conclusions with *EGFP* and *MDR1.* Our results, taken together with previously published reports concerning the molecular mechanisms underlying RNA processing through SS, CTE, or WPRE, provide general guidelines for the construction of improved expression vectors for experimental or medical purposes. In addition, such a synopsis suggests the presence of a functional hierarchy in cellular decisions on RNA processing, in which nuclear export and efficiency of translation constitute the major rate limiting steps.

Obviously, a transcription unit harboring active nuclear retention sequences requires the presence of an export system like Rev/RRE or CTE (reviewed by 8, 14). Rev dependence can be abrogated by mutagenesis of the retention signals located in the coding region, thereby overcoming nuclear retention (25). Our data reveal that the presence of SS (unpublished data) or of the WPRE is not sufficient to compensate for the absence of a CTE. This result is not necessarily in conflict with earlier findings that the WPRE-related element of human hepatitis virus can functionally substitute for Rev/RRE in transient transfection assays (17, 38). These authors used a Rev-dependent reporter which was present in many non-integrated plasmid copies per transfected cell. Our experimental system used to investigate the impact of RNA elements for the expression of HIV-1 *gag* involved generation of retroviral vectors and subsequent expression in retrovirally transduced target cells, thereby enhancing the differences in efficiency of the RNA elements. Moreover, RNA processing of transgenes present in a chromosomal location and in limited copy numbers might be different from that of transgenes which are present in multiple, and sometimes multimerized, episomal copies. Accordingly, the WPRE is not able to substitute for Rev in a Rev minus HIV-1 proviral clone (H.W. and H.G.K., unpublished data), indicating that the results achieved with our experimental approach are predictive for more complex viral systems, including replication-competent lentiviruses. The finding that HIV-1 Gag can be expressed to high levels from a murine leukemia virus-based retroviral vector containing an oligomeric CTE may also have interesting implications for the engineering of improved lentiviral packaging cells.

Interestingly, the presence of a CTE improved RNA expression only in context of β-*globin* and *gpII,* but not for *EGFP* and *MDR1,* except in the absence of SS in the 5′UTR. The observation that SS per se promote RNA export, and thereby also improve viral titers (23), is consistent with recent evidence that splicing is biochemically linked to RNA export by formation of a specific nucleoprotein complex whose precise components remain to be determined (3). As shown in Fig. 4A, the presence of a splice donor already enhanced transcript expression, conceivably by stabilizing the transcript via binding the U1 complex and subsequently other components of the spliceosome (39). Thus it appears that transcripts lacking nuclear retention signals can be efficiently directed into nucleocytoplasmic transport via the splicing machinery, while a CTE mainly overcomes nuclear retention. Moreover, the CTE-specific strong accumulation of the unspliced *β-globin* RNA indicates that this element may act at an early stage of pre-mRNA processing, before completion of the splicing reaction.

In addition, our data obtained with *EGFP* reveal that splicing of the 5′UTR, but not presence of a CTE-related element, improves translation efficiency (Table 1). Underlying mechanisms might be that ribosomal scanning of the unspliced leader region of a retroviral vector is a relatively ineffective and potentially error-prone process (23, 40) or that splicing in the 5′UTR adds factor(s) capable of directing translation initiation (4).

Among the three modules tested, the WPRE had the most uniform and generally—with the notable exception of the CTE-dependent *gag* message—strongest capability of promoting transgene expression. Possibly, the WPRE functions by stimulating different steps of RNA processing, including polyadenylation and RNA export (2, unpublished data cited in 20). Our study indicates that WPRE and CTE share only little functional homology, and therefore possibly involve different nuclear partners, which are known in part for CTE (10, 11), but yet unknown for WPRE (2, 20, 41).

In addition, we reproducibly noted that the WPRE produced an even stronger increase in protein than in RNA levels, consistent with the proposed function of this element not only in nuclear RNA processing and export, but also in translation (2, 20). This could be mediated through improved polyadenylation of RNA, which directly or indirectly promotes translation and RNA stability (reviewed by 42). Our data as well as the work by Zufferey *et al.* (19) also suggest that the major

effect exerted by the WPRE occurs independent of splicing. However, using retroviral vectors that are distinguished by precise alterations of retroviral SS, we obtained evidence that there may be considerable overlap in the function of SS and WPRE, most likely in RNA export. This conclusion is based on our finding that the WPRE acted twofold stronger on RNA and protein expression of a splice-defective transgene compared to the same transgene harboring SS in the 5'UTR. Despite this partial functional redundancy SS and WPRE showed strong additive effects, resulting in highly significant enhancement of expression in case of *EGFP, MDR1,* and, most strikingly, *β-globin.*

In the context of transgenic *β-globin* expressed from a retroviral vector, both the CTE and the WPRE were able to stimulate accumulation of unspliced RNA, thus extending previous observations achieved with the WPRE in transient transfection systems (2, 18, 21). However, our data clearly show that the combination of retroviral SS in the 5'UTR with a WPRE in the 3'UTR is far more efficient for *β-globin* expression than the combination of SS and CTE or each element alone. This may have important implications for new generations of vectors designed for gene therapy of hemoglobinopathies (43, 44).

In summary, it can be concluded that the correct choice of posttranscriptional enhancer modules depends on the type of cDNA to be expressed. Introduction of a CTE-related element can only be recommended when a transcription unit contains sequences that actively inhibit nuclear export, such as nuclear retention signals, or in cases were SS are completely absent. In all other cases, including clinically relevant genes such as *β-globin* and *MDR1,* the combination of SS in the 5'UTR with a WPRE in the 3'UTR produced the most significant enhancement of expression. Further enhancement might be achieved with oligomerization of WPRE sequences; therefore, it was promising to note in the present study that oligomerization of another highly structured RNA element, the CTE of MPMV, was compatible with viral reverse transcription. Thus, our study demonstrates that proper combinations of RNA elements can be identified for a given cDNA to adjust its expression from retroviral vectors to effective and probably therapeutic levels.

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REFERENCES

[1]Jackson, D. A., Pombo, A., and Iborra, F. (2000). The balance sheet for transcription: An analysis of nuclear RNA metabolism in mammalian cells. *FASEB J.* **14**: 242–254.

[2]Huang, Y., Wimler, K. M., and Carmichael, G. G. (1999). Intronless mRNA transport elements may affect multiple steps of pre-mRNA processing. *EMBO J.* **18**: 1642–1652.

[3]Luo, M. J., and Reed, R. (1999). Splicing is required for rapid and efficient mRNA export in metazoans. *Proc. Natl. Acad. Sci. USA* **96**: 14937–14942.

[4]Matsumoto, K., Wassarman, K. M., and Wolffe, A. P. (1998). Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. *EMBO J.* **17**: 2107–2121.

[5]Legrain, P., and Rosbash, M. (1989). Some cis- and trans-acting mutants for splicing target pre-mRNA to the cytoplasm. *Cell* **57**: 573–583.

[6]Chang, D. D., and Sharp, P. A. (1989). Regulation by HIV Rev depends upon recognition of splice sites. *Cell* **59**: 789–795.

[7]Huang, Y., and Carmichael, G. G. (1996). A suboptimal 5' splice site is a cis-acting determinant of nuclear export of polyomavirus late mRNAs. *Mol. Cell. Biol.* **16**: 6046–6054.

[8]Pollard, V. W., and Malim, M. H. (1998). The HIV-1 Rev protein. *Annu. Rev. Microbiol.* **52**: 491–532.

[9]Bray, M., *et al.* (1994). A small element from the Mason–Pfizer monkey virus genome makes human immunodeficiency virus type 1 expression and replication Rev-independent. *Proc. Natl. Acad. Sci. USA* **91**: 1256–1260.

[10]Kang, Y., and Cullen, B. R. (1999). The human Tap protein is a nuclear mRNA export factor that contains novel RNA-binding and nucleocytoplasmic transport sequences. *Genes Dev.* **13**: 1126–1139.

[11]Gruter, P., *et al.* (1998). TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. *Mol. Cell* **1**: 649–659.

[12]Tabernero, C., Zolotukhin, A. S., Bear, J., Schneider, R., Karsenty, G., and Felber, B. K. (1997). Identification of an RNA sequence within an intracisternal-A particle element able to replace Rev-mediated post-transcriptional regulation of human immunodeficiency virus type 1. *J. Virol.* **71**: 95–101.

[13]Wodrich, H., Schambach, A., and Krausslich, H. G. (2000). Multiple copies of the Mason–Pfizer monkey virus constitutive RNA transport element lead to enhanced HIV-1 Gag expression in a context-dependent manner. *Nucleic Acids Res.* **28**: 901–910.

[14]Wodrich, H., and Kraeusslich, H. G. (2000). Nucleocytoplasmic RNA transport in retroviral replication. In *Results and Problems in Cell Differentiation: Cell Polarity and Subcellular RNA Localization* (D. Richter, Ed.). Springer-Verlag. Heidelberg, New York.

[15]Huang, Y., and Carmichael, G. G. (1997). The mouse histone H2a gene contains a small element that facilitates cytoplasmic accumulation of intronless gene transcripts and of unspliced HIV-1-related mRNAs. *Proc. Natl. Acad. Sci. USA* **94**: 10104–10109.

[16]Liu, X., and Mertz, J. E. (1995). HnRNP L binds a cis-acting RNA sequence element that enables intron-dependent gene expression. *Genes Dev.* **9**: 1766–1780.

[17]Huang, Z. M., and Yen, T. S. (1994). Hepatitis B virus RNA element that facilitates accumulation of surface gene transcripts in the cytoplasm. *J. Virol.* **68**: 3193–3199.

[18]Donello, J. E., Loeb, J. E., and Hope, T. J. (1998). Woodchuck hepatitis virus contains a tripartite post-transcriptional regulatory element. *J. Virol.* **72**: 5085–5092.

[19]Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999). Woodchuck hepatitis virus post-transcriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73**: 2886–2892.

[20]Loeb, J. E., Cordier, W. S., Harris, M. E., Weitzman, M. D., and Hope, T. J. (1999). Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus post-transcriptional regulatory element: Implications for gene therapy. *Hum. Gene Ther.* **10**: 2295–2305.

[21]Huang, Z. M., and Yen, T. S. (1995). Role of the hepatitis B virus post-transcriptional regulatory element in export of intronless transcripts. *Mol. Cell. Biol.* **15**: 3864–3869.

[22]Krall, W. J., *et al.* (1996). Increased levels of spliced RNA account for augmented expression from the MFG retroviral vector in hematopoietic cells. *Gene Ther.* **3**: 37–48.

[23]Hildinger, M., Abel, K. L., Ostertag, W., and Baum, C. (1999). Design of 5' untranslated sequences in retroviral vectors developed for medical use. *J. Virol.* **73**: 4083–4089.

[24]Schwartz, S., Campbell, M., Nasioulas, G., Harrison, J., Felber, B. K., and Pavlakis, G. N. (1992). Mutational inactivation of an inhibitory sequence in human immunodeficiency virus type 1 results in Rev-independent gag expression. *J. Virol.* **66**: 7176–7182.

[25]Schneider, R., Campbell, M., Nasioulas, G., Felber, B. K, and Pavlakis, G. N. (1997). Inactivation of the human immunodeficiency virus type 1 inhibitory elements allows Rev-independent expression of Gag and Gag/protease and particle formation. *J. Virol.* **71**: 4892–4903.

[26]Buchman, A. R., and Berg, P. (1988). Comparison of intron-dependent and intron-independent gene expression. *Mol. Cell. Biol.* **8**: 4395–4405.

[27]Mergener, K., Facke, M., Welker, R., Brinkmann, V., Gelderblom, H. R., and Krausslich, H. G. (1992). Analysis of HIV particle formation using transient expression of subviral constructs in mammalian cells. *Virology* **186**: 25–39.

[28]Ratner, L., *et al.* (1987). Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res. Hum. Retroviruses* **3**: 57–69.

[29]Knipper, R., *et al.* (2000). Improved post-transcriptional processing of an MDR1-retrovirus elevates expression of multidrug resistance in primary human hematopoietic cells. *Gene Ther.*, in press.

[30]Jaeger, J. A., Turner, D. H., and Zuker, M. (1989). Improved predictions of secondary structures for RNA. *Proc. Natl. Acad. Sci. USA* **86**: 7706–7710.

[31]Jaeger, J. A., Turner, D. H., and Zuker, M. (1990). Predicting optimal and suboptimal secondary structure for RNA. *Methods Enzymol.* **183**: 281–306.

[32]Kinsella, T. M., and Nolan, G. P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther.* **7**: 1405–1413.

[33]Zolotukhin, A. S., Valentin, A., Pavlakis, G. N., and Felber, B. K. (1994). Continuous propagation of RRE(–) and Rev(–)RRE(–) human immunodeficiency virus type 1 molecular clones containing a cis-acting element of simian retrovirus type 1 in human peripheral blood lymphocytes. *J. Virol.* **68**: 7944–7952.

[34]Arai, T., Takada, M., Ui, M., and Iba, H. (1999). Dose-dependent transduction of vesicular stomatitis virus G protein-pseudotyped retrovirus into human solid tumor cell lines and murine fibroblasts. *Virology* 260: 109–115.

[35]Sorrentino, B. P., *et al.* (1992). Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human MDR1. *Science* 257: 99–103.

[36]Eckert, H. G., *et al.* (1996). High-dose multidrug resistance in primary human hematopoietic progenitor cells transduced with optimized retroviral vectors. *Blood* 88: 3407–3415.

[37]Baum, C., Hegewisch-Becker, S., Eckert, H. G., Stocking, C., and Ostertag, W. (1995). Novel retroviral vectors for efficient expression of the multidrug resistance (mdr-1) gene in early hematopoietic cells. *J. Virol.* 69: 7541–7547.

[38]Donello, J. E., Beeche, A. A., Smith, G. J., III, Lucero, G. R., and Hope, T. J. (1996). The hepatitis B virus post-transcriptional regulatory element is composed of two subelements. *J. Virol.* 70: 4345–4351.

[39]Hammarskjold, M. L., Li, H., Rekosh, D., and Prasad, S. (1994). Human immunodeficiency virus env expression becomes Rev-independent if the env region is not defined as an intron. *J. Virol.* 68(2): 951–958.

[40]Berfioz, C., and Darlix, J. L. (1995). An internal ribosomal entry mechanism promotes translation of murine leukemia virus gag polyprotein precursors. *J. Virol.* 69: 2214–2222.

[41]Smith, G. J., III, Donello, J. E., Luck, R., Steger, G., and Hope, T. J. (1998). The hepatitis B virus post-transcriptional regulatory element contains two conserved RNA stem–loops which are required for function. *Nucleic Acids Res.* 26: 4818–4827.

[42]Colgan, D. F., and Manley, J. L. (1997). Mechanism and regulation of mRNA polyadenylation. *Genes Dev.* 11: 2755–2766.

[43]Bender, M. A., Miller, A. D., and Gelinas, R. E. (1988). Expression of the human beta-globin gene after retroviral transfer into murine erythroleukemia cells and human BFU-E cells. *Mol. Cell. Biol.* 8: 1725–1735.

[44]Emery, D. W., and Stamatoyannopoulos, G. (1999). Stem cell gene therapy for the beta-chain hemoglobinopathies. Problems and progress. *Ann. N. Y. Acad. Sci.* 872: 94–107; discussion 107–108.